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**DNA Binding Activities in Cerebellar Granule Cell Neurons
Recognizing the Promoter for
The GABA_A- α 6 Receptor Subunit**

A Thesis
Submitted to the Faculty
Of The
WORCESTER POLYTECHNIC INSTITUTE
in partial fulfillment of the requirements for the
Degree of Master of Science
In
Biology
By

Rachel E. Stock

August 21, 2002

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ABSTRACT

The objective of this thesis project was to begin identifying which regulatory transcription factors are involved in the up-regulation of the gene promoter for the $\alpha 6$ subunit of the gamma-alpha-butyric acid (GABA_A- $\alpha 6$) receptor in cerebellar granule cell neurons (GCNs). Although a 150 base pair sequence proximal to the GABA_A- $\alpha 6$ gene promoter had been characterized previously using electrophoretic mobility shift assays (EMSAs), the specific transcription factor(s) needed to express the GABA_A- $\alpha 6$ gene had not been examined.

This project utilized EMSAs to investigate this 150 base pair sequence further. It was found that when this sequence proximal to the gene promoter was divided into two overlapping halves, both shortened sequences were able to compete for binding with nuclear extracts. The full-length sequence was further divided into six sub-regions, and double-stranded competitors were generated from synthetic oligonucleotides. The only oligonucleotide to compete was the one that corresponded to the region of overlap between the left and right halves. This overlap region contains consensus sites for OCT-1, STAT, and the regulatory transcription factor NF-1. An NF-1 consensus sequence was able to compete DNA-protein complexes. Supershift assays showed that a xenopus NF-1 antibody, previously shown to compete in gel shift assays, caused a mobility shift of the DNA-probe complex. Analysis of extracts from granule cell neurons, cultured from 0 to 6 days *in vitro* (DIV) indicated NF-1 to be present all time points. Northern analyses were performed using probes for NF-1A, NF-1B, NF-1C and NF-1X. NF-1A transcripts were observed from 0 to 6 DIV, while NF-1B and NF-1X transcripts were present at 2 and 4 DIV. NF-1C RNA was barely detectable at any time point.

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BACKGROUND

Granule cell neurons

The cerebellum is the center of motor control and balance in the brain, and may be involved in some higher cognitive functions. In transgenic mouse models where cerebellar development is disrupted, the surviving animals typically have dysfunctional voluntary movement (Eisenman and Brothers, 1998). The cerebellum consists of several cell types: granule cell neurons, Purkinje cells, and glial cells. Granule cell neurons (GCNs) are important elements in the brain, comprising the largest percentage of the cell population in the adult human cerebellum (Jones et. al, 2000). GCNs are significant as major components of the cerebellum and in their involvement in coordination and movement. The events that contribute to the unique identity of GCNs are particularly interesting. The nervous system is made up of many diverse neuronal cell types. Knowledge gained by studying GCN precursors, and the molecular events that determine GCN identity, can be applied to further the understanding of the differentiation and development of other types of neurons.

Cerebellar granule cell precursors start off in the rhombic lip, which is derived from the roofplate of the fourth ventricle during embryonic development. These cells originate from both the early midbrain and hindbrain, and studies have pointed to rhombomere 1 as the sole source of the rhombic lip. Granule cell precursors migrate away from the roofplate to form the external granule layer (EGL) and express molecular markers (*Math1*, *Zipro1*) specific for differentiation. Further patterning of GCNs may be

signaled by cell-cell induction, or regulated by chemorepellent and/or spatial-temporal factors (Wingate, 2001). GCNs begin their terminal differentiation in the external granule layer (EGL), and then migrate to the internal granule layer (IGL) (Figure 1).

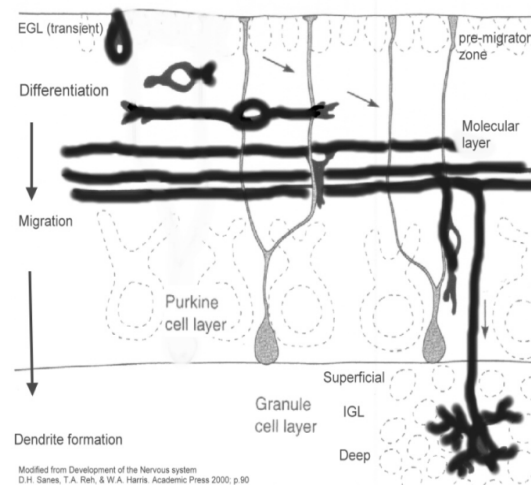


Figure 1. GCN differentiation and migration. Abbreviations: EGL= External Granule Layer; IGL= Internal Granule Layer. Original from Sanes,et al., 2000, Page 90. Development of the Nervous System, Academic Press Copyright 2000.

Before migration begins, projections known as parallel fibers are formed. The parallel fibers synapse with Purkinje cells and mossy fibers, and extend further with time as the molecular layer forms from the deep EGL and pre-migratory zone. Terminally differentiating GCNs express the genes required for migration to the IGL, such as *Unc5H3*, and *Netrin-1* (Wingate, 2001). These genes are believed to play a role in positional identity of neurons in the prospective optic chiasm, and are involved in early segmentation of the forebrain in mouse. Similar genes may be involved in determining the cell fate of the cerebellar cell precursors (Hatini et. al, 1994). The gene of the GABA_A- $\alpha 6$ receptor subunit becomes expressed at the completion of GCN differentiation and migration, when these cells have reached the IGL of the cerebellum

(Figure 1). This occurs in mice during the first two to three weeks of postnatal life, as dendrites and synapses form (Jones et. al, 2000).

GABA_A- α 6 Receptor Subunit

Gamma-alpha-butyric acid (GABA) is an inhibitory neurotransmitter, that regulates GCN function by negatively controlling neuronal stimulation. Inhibition of GCNs takes place as a result of GABA binding to its receptors, either through isolated pulses of GABA where it acts on synaptic receptors, or by continuous signaling of GABA receptors in the extrasynaptic space, resulting in sustained inhibition. The extrasynaptic receptors have a higher sensitivity or affinity for GABA and are not desensitized by the neurotransmitter being present for extended periods of time. This feature makes extrasynaptic receptors exceptional mediators of tonic (sustained) inhibition (Soltez and Nusser, 2001), thus keeping the neuron from firing. The receptor protein is made up of subunits, α , β , γ , δ , and ϵ . The subunits exist in various isoforms, such as the α 6 subunit. The GABA_A- α 6 and δ -subunits appear to be closely linked and are located only in receptors outside synapses, but receptors which include the γ 2 subunit are concentrated inside synapses.

GABA receptors may be vital in the development of the central nervous system, although receptor activation regulates GABA receptor subunit gene expression regardless of age (Russek et. al, 2000). Additionally, expression of α 6 can be induced by electrical activity, independent of stimulation by the glutamate receptor (Mellor et. al, 1998). The functioning of chemical synapses requires the expression of specific receptor subunits, which in turn need to be precisely regulated to control neuronal phenotype. It is believed

that there is some built-in redundancy with the GABA receptor subunits, but the compensation effects are limited by the fact that specific subunits tend to co-localize. When temporal lobe epilepsy was induced in rats with pilocarpine injections, the distribution of the GABA receptor subunits was altered before and during the onset of chronic epilepsy. $\alpha 1$ subunit mRNA expression was significantly reduced, but $\alpha 4$ expression was increased. Expression of δ - and ϵ -subunits was also increased in epileptic dentate granule cells (Brooks-Kayal et. al, 1998). Excitatory signals modulate GCNs by a stimulatory input. GCNs lacking the $\alpha 6$ - and δ -subunits of the GABA_A receptor do not exhibit this type of sustained conductance. Granule cell excitability is regulated by GABA, but is absent in $\alpha 6^{-/-}$ mice even though $\alpha 6^{-/-}$ mice demonstrate typical motor function. The expression of the K⁺ channel TASK-1 is increased 20% in $\alpha 6^{-/-}$ mice and seems to compensate for the missing $\alpha 6$ subunits by changing K⁺ channel leak conductance through the cell membrane (Brickley et. al, 2001).

When Jones et. al (1997) created a GABA_A- $\alpha 6$ knockout mouse, they made the following observations: 1) Binding of the Ro15-4513 ligand, which binds GABA receptors containing $\alpha_x\beta\gamma_2$ subunits, is absent in $\alpha 6^{-/-}$ mice. 2) The δ subunit of the GABA receptor is almost completely lost in $\alpha 6^{-/-}$ mice, and this loss takes place after protein translation. The δ subunit appears to be preferentially bound to $\alpha 6$, and therefore disrupting $\alpha 6$ also affects the expression of the δ subunit. 4) $\alpha 1$ subunit does not compensate for the loss of $\alpha 6$ (Jones et. al, 1997).

Immunoprecipitation and ligand-binding experiments carried out by the Nusser group determined the fate of other GABA_A subunits in granule cell neurons when the $\alpha 6$ subunit gene was disrupted. The levels of δ , $\beta 2$, and $\beta 3$, decreased by 77%, 53%, and

21% respectively. The expression of $\gamma 2$ and $\alpha 1$ was reduced by 41% and 27%. The conclusion was that there are no compensating changes in expression of these subunits to make up for the loss of $\alpha 6$. Because of the observed decrease, they also concluded that the $\alpha 6$ protein is linked to the formation of oligomers with β subunit protein and its surface expression as well (Nusser et. al, 1999).

Importance of understanding GABA_A- $\alpha 6$ gene regulation

The GABA_A- $\alpha 6$ receptor subunit is a unique marker of terminally differentiated GCNs, and its detection by immunocytochemistry coincides with the timing of dendrite formation. This timing enables the study of cell-specific events in GCNs, and is also a useful tool for modeling dendritogenesis. The specific regulatory factors for activation of the gene promoter have not yet been defined. Although the elements of gene up-regulation may not be the same in every case, the study of regulatory factors involved in GABA_A- $\alpha 6$ expression may serve as a model for other GABA subunit genes. Exploring such regulatory factors may in turn reveal insights into the molecular means of determining cell specificity as well as synapse and dendrite formation. The information gathered by studying transcription factors may also shed light on the mechanisms of terminal differentiation and neuronal plasticity. Also, by understanding GABA_A- $\alpha 6$ gene transcription, a relationship of subunit assembly pathways to subunit co-localization may be uncovered (Jones et. al, 1997).

The Jones group (2000) used a “knock-in” strategy to create transgenic mice that express lacZ (β -galactosidase) under the control of the GABA_A- $\alpha 6$ promoter in GCNs. The β -galactosidase expression cassette was inserted into exon 8 by homologous

recombination, using an internal ribosome entry site (IRES). They then used the expression of LacZ in $\Delta\alpha6$ lacZ mutant mice to follow the pattern of GABA $\alpha6$ subunit expression in postnatal day 5- to 10 (P5-P10) mice. They found that $\alpha6$ gene expression begins in the deep cerebellar layers of lobule X and parts of IX, where the first GCNs migrate. This “inside-out” pattern is initially bi-layered, with only the deeper cells expressing $\alpha6$. The expression pattern resembled the combined patterns of the transcription factors Otx1 and Otx2 (Jones et. al, 2000) and also that of *Engrailed-2* (En-2). En-2 expression precedes that of $\alpha6$ by 1.5 weeks, and is believed to prompt spatial cues during cerebellum development by separating it into a network of positional information necessary for patterns of folding and afferent connections (Millen et. al.1995). It is theorized that whatever transcription factor activates $\alpha6$ may interact with En-2. No single agent has been uniquely identified to ultimately change the $\alpha6$ gene expression pattern; although electrical activity, brain-derived neurotropic factor, and cAMP are known to affect the level $\alpha6$ expression (Millen et. al.1995). While it is postulated that transcriptional activation may come from external signals, $\alpha6$ induction appears to be intrinsically programmed into GCNs (Jones et. al, 2000).

GABA $_A$ - $\alpha6$ subunit promoter: prior studies

The DNA sequences of the GABA $_A$ - $\alpha6$ subunit gene and the regions proximal to and upstream of the promoter region have been identified (Jones, et. al. 1997). Previous transgenic analysis of the promoter regulation used LacZ reporter constructs derived from a knockout mouse. The Bahn group constructed a transgene containing an internal ribosome entry site and a LacZ reporter. Using this transgene, they determined that the 5’

end of the $\alpha 6$ subunit gene directs granule-cell specific gene expression. When the transgene was expressed, no β -galactosidase staining was observed in the cerebellum (Bahn et. al, 1997). This research established that the timing of GABA_A- $\alpha 6$ gene up-regulation, as well as cell specificity is transcriptionally regulated. These experiments set the groundwork for transgene deletion experiments in the Kilpatrick lab, where the same construct was used to produce transgenic mice, as well as for transient co-transfections. Deletion analysis of various transgene promoter constructs was already an ongoing project. Transgene analysis complements the transient transfection studies, since a transgene is stably expressed, and relates directly to *in vivo* cell physiology, requiring chromatin and histones, as well as exposure to necessary developmental influences for gene transcription to occur.

The McLean group (2000) cloned and sequenced the proximal 5' flanking regions of the mouse GABA_A- $\alpha 6$ subunit gene (Figure 2). Through the use of PCR constructs, primary neuronal culture, and transient transfections/reporter gene assays they identified a major transcriptional initiation site, and they determined that a 155 base-pair TATA-less proximal promoter can drive GCN cell specificity. Transient transfection studies showed that this minimal promoter region contributes to GCN cell specificity. The homologies in the consensus regulatory sequences in mouse, rat, and human point to a similar mechanism of cell-specific expression. There is greater than 90% conservation between mouse, rat and humans in the sequence of the proximal promoter of the GABA_A- $\alpha 6$ subunit gene. This suggested that the DNA regulatory elements involved in $\alpha 6$ subunit gene transcription are also conserved during evolution.

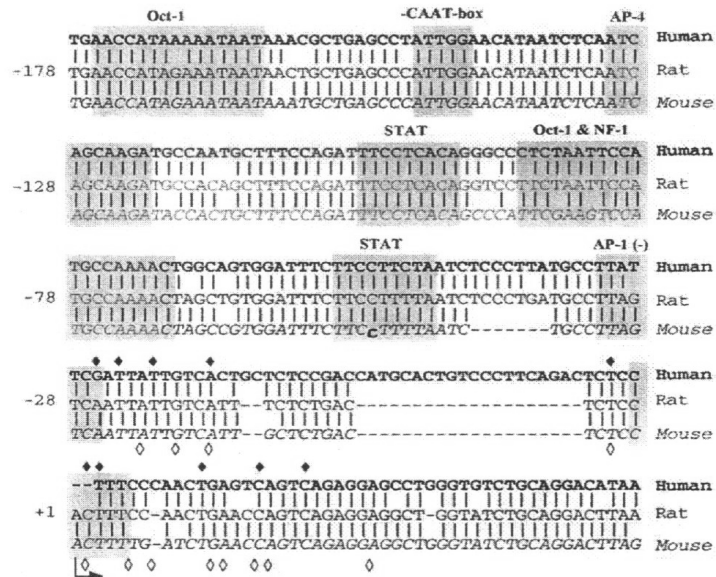


Figure 2. Alignment of the human, rat and mouse GABA_A-α6 proximal promoter regions. This figure compares the promoter sequences for the GABA_A-α6 subunit genes for human, rat, and mouse. Original figure from: McClean, et al., 2000.

These upstream sequences may encode binding sites for transcription factors that are known or share homology to known transcription factors, or may be novel. These studies implicate regulatory sequences upstream of the initiation region, which encode binding sites for transcription factors related to activation of the GABA_A-α6 subunit gene. Although neuronal-specific genes are often directed by negative regulation, deletion analysis in the GABA_A-α6 minimal promoter implied that certain regions contain positive regulatory element(s) that may drive cell specificity (McLean et.al 2000). Figure 2 shows the alignment of the 5' ends of the mouse, rat, and human GABA_A-α6 subunit genes which was used extensively in this project for creating the various gel shift probes and competitors.

PURPOSE

Based on the information from the above prior research, the objective of this thesis was to identify and localize transcription factor binding sites involved in cell-specific regulation of the promoter for the mouse GABA_A- α 6 subunit gene.

The mouse is a widely accepted model for studying homologous gene expression events in other mammal species and has extensively been used for *in vitro* and/or *in vivo* studies. Mouse GCNs are a good model for studying expression events in this receptor subunit, using cell cultures, nuclear extracts and transgenic mice. Relatively large numbers of cells can be prepared in high purity and for cell culture analysis. The model used in this thesis is mouse GCNs cultured *in vitro* for 0 to 6 days. Mouse GCN cell cultures were prepared from postnatal day 6 (P6) pups. Day 0 *in vitro* (0 DIV) is therefore equivalent time-wise to a P6 cell preparation, and cells that survive the culture process are GCN progenitors (D. Kilpatrick, personal communication). By 1 DIV, the cells are postmitotic and differentiating. Expression of the GABA_A- α 6 subunit protein is not detected by immunocytochemistry in these cultures until 6 DIV. For the purposes of this project, P15 cerebellum nuclear extracts were regarded as equivalent time-wise to nuclear extracts from 6 DIV cells.

The primary method chosen for this project was the electrophoretic mobility shift assay (EMSA), also known as a gel shift assay. The EMSA is employed to localize and characterize the sites of protein-DNA complex formation, determine the DNA binding affinity of proteins, and verify the sequence specificity of DNA binding. This assay is useful for identifying the DNA-binding proteins present or absent in different cell types (cell-type specificity) or under different cellular conditions (e.g., developmentally

regulated). These DNA-binding proteins are good candidates for being transcription factors that up- or down-regulate the gene promoter of interest, in this case, the GABA_A- α 6 gene.

The EMSA uses a mixture of radioactively labeled DNA probe and nuclear protein extract for detecting sequence-specific DNA-binding proteins. Using polyacrylamide gel electrophoresis, sequence-specific DNA-binding proteins in a crude protein extract can be detected by comparing the way that free versus bound probe travels through a gel. The observed shift results from the formation of DNA-protein complexes that move more slowly through the gel than non-bound DNA. A competitor assay utilizes excess amount of unlabeled DNA, which decreases via competitive binding the amount of labeled probe bound by protein. The competitors used for this thesis are oligonucleotides used to localize sequence-specific DNA-binding proteins. A supershift assay utilizes antibodies to DNA-binding proteins as competitors. The antibody is added prior to probe, and when the DNA-binding protein of interest is present in the reaction, the antibody causes a mobility shift by binding this protein and shifting the DNA-protein complex to a higher position on the gel. If the antibody used is not supershifting, then it simply disrupts the complex formed with the probe.

While electrophoretic mobility shift assays are able to help identify protein complexes, they are limited in that they may not necessarily reflect events that occur in the living cell. Also, by using an excess of competitor, the binding reaction may not be at equilibrium. The results of a gel shift provide information about binding affinities of the competitor, but it may not be possible to pinpoint this binding in the probe sequence (Little, 2001).

MATERIALS AND METHODS

Probe DNA preparation:

The experiments for this project were based on preliminary EMSA experiments (unpublished results) that used probes made from PCR products that encompass the region of the proximal promoter sequence shown in Figure 2. This proximal promoter sequence was dubbed the “A” region, and that nomenclature was also utilized for this project. The DNA for the “A” probe was generated by PCR. The template DNA used for the PCR reaction was purified from transgene plasmid DNA, mGABA α 6LacZ. The primers used were oligonucleotides from Integrated DNA Technologies, Inc. (Table 1).

Oligonucleotide:	Sequence:
mGABA5'A	GGAATTCAAATGCTGAGCCCATG
mGABA3'A	GGAATTCTGGAGAGTCAGAGCAATG

Table 1. Oligonucleotide sequences used as primers for PCR product for probe “A” synthesis.

These primers contained EcoRI restriction sites, allowing the double-stranded product to be digested for end-labeling. The amplification reaction mixture contained the following: 10 μ l of template DNA (10 ng/ μ l); 10 μ l of thermophilic 10X buffer (Promega Cat#M190A); 8 μ l 25 mM MgCl₂ (Promega Cat#A351B); 5 μ l each of 10 μ M primers mGABA5'A and 3'A; 4 μ l of 5 mM dNTPs; 57 μ l nuclease-free distilled water; and 1 μ l Taq polymerase (Promega Cat#M166A). The thermocycler program was: 94 °C for 2 minutes (1 cycle); 94 °C for 30 seconds, 45 °C/30 sec., 72 °C/1 minute (5 cycles); 94 °C/30 sec., 58 °C/30 sec., 72 °C/1 min. (30 cycles); then 72 °C for 5 min., and a soak

temperature of 5 °C. PCR product was digested with EcoRI (New England Biolabs R0101S) and purified with a PCR Purification Kit (Qiagen Cat#28104) before labeling.

The reaction mixture for making labeled probe contained the following: 50 ng of double-stranded DNA; 2 µl unlabeled dCTP, dGTP and dTTP, 0.2 mM final concentration (Promega Cat#U122A, U121A, U123A); 3 µl α P³² dATP; 1 µl Klenow polymerase (New England Biolabs Cat#M0210S); sterile distilled water to bring the volume to 20 µl. The reaction was incubated at room temperature for 45 minutes, and then excess radioactive dATP was chased with 2 µl of 10 mM of unlabeled dATP (Promega Cat#U120A) for 5 minutes at room temperature. The reaction was stopped with 30 µl of TE buffer (Tris 10 mM, EDTA 1 mM, pH 8.0) and the probe was purified with a Bio-Rad Bio-Spin 6 column (Cat#737-6002). For quantification, 1 µl aliquots of probe were dotted onto Whatman DE81 filter paper (Cat#3658323), before and after washing and purification, and placed in 5 ml of EcoLume Scintillation fluid (ICN Cat#882470). Scintillation counts were then taken to determine the efficiency of labeling and probe concentration.

Competitor Preparation:

To design the competitors for the EMSA experiments, the “A” proximal promoter region was first split into two overlapping ~90 base-pair segments “Aa” and “Ab”. The “A” region was also divided into 6 overlapping regions A1ab through A6ab. (Table 2)

Oligo Name:	Sequence:	Oligo Name:	Sequence:
A1a	ATGCTGAGCCCATTTGGAACA	A4a	AACTAGCCGTGGATTTCTTC
A1b	TTGAGATTATGTTCCAAT	A4b	ATTAAAAGGAAGAAATC
A2a	GATACCACTGCTTTCCAGAT	A5a	TTCCTTTTAATCTGCCTTAGTC
A2b	CTGTGAGGAAATCTGGAAAG	A5b	TGACAATAATTGACTAAGGCA
A3a	TTCCTCACAGCCCATTCGAAGTCC	A6a	AATTATTGTCATTGCTCT
A3b	CTAGTTTTGGCATGGACTTCGAA	A6b	TGGAGAGTCAGAGCAATGA

Table 2. Oligonucleotide sense and antisense sequences used for probe and competitors in EMSA assays.

Double-stranded competitor DNA for the EMSA assays was generated by annealing and filling in oligonucleotide pairs purchased from IDT, Inc. For example, the double-stranded “A3ab” competitor was made using oligonucleotides A3a and A3b. Melting temperatures and stem loop structure were checked using OligoTech software.

The antibodies available do not distinguish NF-1 isoforms. To verify that NF-1 core sequence is responsible for observation with A3, a mutation of the NF-1 consensus site was created using the TESS Program. For the NF-1-Mut1, a simple substitution mutation was made that corresponded to the commercial oligonucleotide mutant from Santa Cruz Biotechnology (within the A3 core). To create NF-1-Mut2, substitution and deletion mutations were used because just doing one or the other resulted in the creation of new consensus sequences for other known transcription factors (Table 3).

NF-1 Consensus oligonucleotide:	NF-1 Mutant oligonucleotide:
5' TTTTGGATTGAAGCCAATATGATAA	5' TTTTGGATTGAATAAAATATGATAA
A3b-NF-1mut1:	A3b-NF-1mut2
CTAGTTTTTTAATGGACTTCG	CTAGTTCGGACTTCG

Table 3. Sequence for the consensus and mutated NF-1 and A3 oligonucleotides.

The reaction mixture for making double-stranded “cold” competitors contained the following: 500 pmoles each of 5’ and 3’ oligonucleotide DNA, in a total volume of 20 μ l, denatured for 5 minutes at 85°C and cooled at room temperature 15 minutes; 7 μ l 10X buffer (New England Biolabs); 7 μ l unlabeled dATP, dCTP, dGTP and ddTTP, (10 mM); 8 μ l α P³² dATP diluted 1:100; 2 μ l Klenow polymerase; 26 μ l sterile distilled water. The reaction was incubated at room temperature for 1 hour, and then the reaction was stopped with 30 μ l of TE buffer and each competitor was purified with a Bio-Rad Bio-Spin 6 column, and 1 μ l aliquots were taken for quantifying as above. Scintillation counts were again taken to determine the labeling efficiency and competitor concentrations.

EMSAs:

All gel shift experiments were carried out according to a general protocol (*Current Protocols in Molecular Biology*). George Gagnon and Daniel Kilpatrick generated all nuclear extracts used for the gel shift experiments from primary cultures of GCNs derived from P6 mouse cerebellum. Polyacrylamide gels were poured fresh on the day of each experiment, and the glass plates were cleaned with 70% ethanol. Five percent polyacrylamide gels contained 6.7 ml of 29:1 acrylamide : bisacrylamide, 1 ml of 10X TBE buffer (Tris-Base 890 mM, Boric Acid 890 mM, EDTA 20 mM), 32.2 ml of distilled water, 300 μ l of 10% ammonium persulfate, and 50 μ l of TEMED. Gels were pre-run at ~20 milliamps at 4°C for at least 30 minutes before loading samples.

The binding reactions contained 1 μ l of 1 μ g/ μ l Poly dI-dC (Amersham Pharmacia Cat#27-7880-01), 1.4 μ l of 10 mM phosphate buffer with 1 mM EDTA, 2 μ l of P^{32} -labeled probe diluted to 30000 cpm, and 1.6 μ l of either sterile water or DNA competitor at a 50-fold excess relative to the probe DNA concentration. To this mixture was added 0.5- to 1.25 μ g of nuclear extract and enough nuclear extraction buffer to equal 7 μ l. This brought the total reaction volume to 14 μ l, with a final NaCl concentration to 100 mM. The reaction tubes were then incubated for 15 minutes on ice before loading on the gel. Gels were run for approximately 1 hour at 20 milliamps, and then dried on Whatman paper for 1 hour on a vacuum gel dryer. The dried gels were then exposed overnight in a cassette to Kodak X-Omat X-ray film at -80°C .

Northern analysis

All equipment used for RNA and Northern gel preparation (e.g.: pipettes, tips, glassware, and gel apparatus) was for exclusive RNA work, and was pre-treated with 0.1% DEP-C, RNase-AwayTM and/or rinsed with 0.1% DEP-C water. RNA was extracted from cultured granule cell neurons on days 0, 2, 4, and 6 using Tri Reagent (Sigma), according to the manufacturer's directions. RNA samples were precipitated in sodium acetate and ethanol. RNA samples were prepared for electrophoresis using 5 μ g RNA, 10 μ l of deionized formamide, 2.5 μ l of 10X MOPS buffer (MOPS 200 mM, sodium acetate 100 mM, EDTA 10 mM pH 7.0), 3 μ l of 37% formaldehyde, and 0.1% DEP-C water to a volume of 26.5 μ l. The samples were denatured for 5 minutes at 68°C , and then 1 μ l of ethidium bromide (diluted 1:6) and 2.5 μ l of RNA loading dye (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, and 0.4% xylene cyanol) were added. The prepared samples were run on a 1% agarose/formaldehyde denaturing gel for 2 hours

at 200 volts. The gel was transferred with 6X sodium phosphate buffer (pH 7.8) onto a nylon GeneScreen Plus membrane (NEN Life Sciences, Cat#NEF-976) overnight, then photographed and baked at 80°C for four hours to crosslink the RNA.

Rat NF-1 cDNA splice variants were generously provided by Masayoshi Imagawa and cloned into pBluescript KS. Probe DNAs for NF-1B, -C, and -X were amplified, excised, digested and purified by Debra Mullikin-Kilpatrick. Colonies were grown on LB-amp plates overnight, and then amplified in LB-amp medium overnight. DNA was extracted using a Maxi-Prep kit (Qiagen). NF-1A cDNA was digested with restriction enzyme Afl-II, yielding a 633 base-pair fragment. NF-1B cDNA was digested with restriction enzyme KpnI, yielding a ~600 base-pair fragment. NF-1C cDNA was digested with restriction enzymes BglII and BstEII, yielding a 583 base-pair fragment. NF-1X cDNA was digested with restriction enzymes Afl-II and BglII, yielding a 600 base-pair fragment. DNA was run on a 1% agarose gel and purified using a Gel Extraction Kit (Qiagen Cat#28704). The nucleotide sequences for NF-1A, NF-1B, NF-1C, and NF-1X can be found in Chaudhry et.al., 1997.

Probes were prepared as follows: 25 ng DNA was brought to a volume of 30 µl with nuclease-free water and was denatured for 2 minutes at 95°C. The following were then added in order: 10 µl of 5X labeling buffer; 2 µl of unlabeled dNTPs (minus dATP) at 500 µM; 5 µl of nuclease-free bovine serum albumin; 5 µl α P³²-labeled dATP; 1 µl Klenow polymerase. The reaction mixture was incubated for 1 hour at room temperature, and then 2 µl of 0.5 M EDTA was added to stop the reaction. The probe was purified with a Bio-rad Biospin 6 column, and 1 µl aliquots were dotted onto Whatman paper and counted in Ecolume scintillation fluid in a scintillation counter. 2×10^6 cpm/ml of probe

were used for each hybridization. The membranes were incubated on a rotator in a hybridization oven at 45°C for 3 hours in pre-hybridization solution: 15 ml of 50% DI formamide; 7.5 ml of 0.05 M sodium phosphate buffer, pH 7.0; 0.6 ml of 1X Denhardt's solution; 7.5 ml of 5X SSC; 3.0 ml of 1% SDS; and 250 µl of sodium heparin. Probe was added to fresh hybridization solution before returning to the oven for overnight incubation at 45°C. The membranes were rinsed in increasing stringency of SSC buffer (2X, 0.2X, 0.02X) and exposed in a cassette to X-ray film for 4 days to 1 week.

RESULTS

Previous Probe “A” analysis:

The proximal promoter described by McClean, et. al.(2000) was analyzed with a variety of fragments in order to localize the binding activity observed in earlier EMSA experiments. These previous experiments found a single major complex formed with nuclear extracts from cerebellum and a probe for the proximal 5' flanking region, spanning ~150 base pairs (Figure 3).

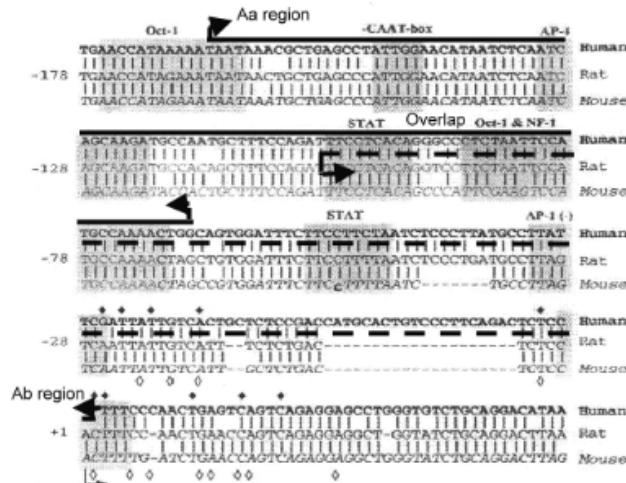


Figure 3. The proximal promoter sub-divided into “Aa” and “Ab”. The solid line spans the sequence of the “Aa” region and the dotted line spans the sequence of the “Ab” region. Also noted is the sequences overlap. Modified from: McClean, et al., 2000

With nuclear extracts from cultured GCNs, a major complex and a smaller, nonspecific complex was observed in gel shifts. Experiments done prior to this thesis project focused on regional tissue specificity, comparing Day 15 cerebellum to cortex, and GCNs cultured 6 days *in vitro* (6 DIV), when expression of GABA_A-α6 subunit is known to be present. Both the Aa and Ab oligonucleotides competed equally well to the

major complexes. The results indicated either similar transcription factor binding sites on both regions, or a single binding region within the overlapping sequences for Aa and Ab. This earlier research also determined that the oligonucleotide that corresponded to the D-hand region did not compete with the synthetic probe “A” (unpublished results). This was used in subsequent experiments as a negative control. Knowing that the complexes were cell-specific, the next step was to narrow down the sequence(s) responsible for the DNA-protein complex formation.

Sub-regions A1 to A6

The “A” proximal promoter region was sub-divided into six shorter sequences, and double-stranded competitors A1 to A6 were generated by using oligonucleotide pairs in a filling-in reaction (Figure 4).

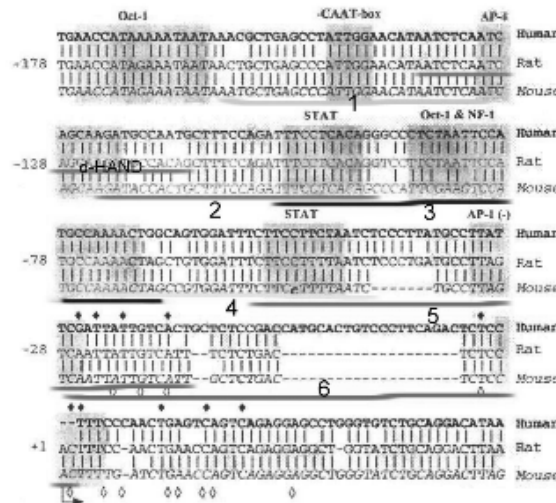


Figure 4. Map showing the sequences of the double-stranded competitors A1(1) through A6(6) used for “A” region analysis.

The “a” oligonucleotide was used as the template for the “sense” strand, and the “b” oligo was used as the reverse complement for the double-stranded competitor

synthesis (Figure 4). The first experiment carried out was a gel shift using the full-length “A” probe in a competition experiment with these six duplex oligonucleotides. Nuclear extracts from 6 DIV GCN were used. Competition was only observed with the A3 oligonucleotide (Figure 5). This A3 sequence corresponds to the 37 base pair overlap between the Aa and Ab sub-regions.

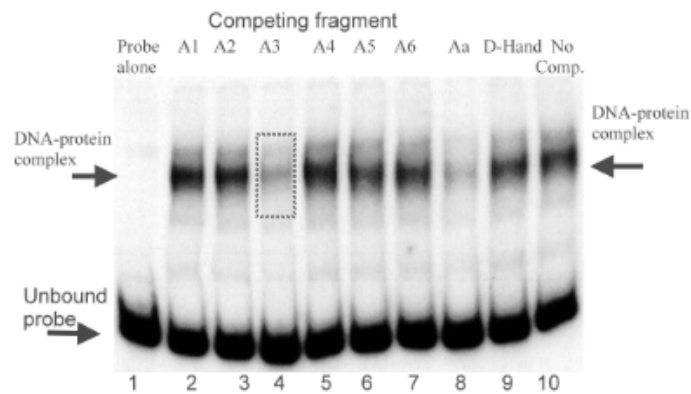


Figure 5. EMSA showing the experiment with probe “A” competitors A1 through A6. Arrows indicate the major complex formed with probe “A” and 6 DIV GCN nuclear extracts. The dotted-line box outlines the diminished signal caused by competition with A3 in lane 4. This same shift is seen with the Aa positive control competitor in lane 8. D-Hand in lane 9 is a negative control. Lane 10= no competitor

NF-1 Competition with GCN 6DIV

There are three transcription factor consensus sequences encoded in the proximal promoter where the A3 oligonucleotide competes (McClean et. al, 2000). These are NF-1, OCT-1, and STAT. The OCT-1 consensus sequence is not conserved between mouse, rat, and human, and it was decided to be the least likely candidate as a specific, regulatory transcription factor for the GABA_A-α6 promoter. The STAT consensus sequence is near the end of the overlap between the Aa and Ab subregions and would have been chosen second, because binding is less likely when the consensus site resides

at the end of a sequence. The NF-1 transcription factor consensus sequence was selected as the first to try in gel shift competition experiments.

This EMSA experiment used a commercially available NF-1 consensus oligonucleotide and a mutated oligonucleotide from Santa Cruz Biotechnology, Inc. (catalog #sc-2553 and #sc-2554). Figure 6 shows that the NF-1 consensus oligonucleotide completely competes the 6DIV GCN DNA-protein complexes, and at equimolar concentrations the competition is stronger than the full-length “A” competitor. This indicated stronger binding affinity for NF-1 than for the full-length probe. The mutated oligonucleotide does not compete. It is possible that the full-length “A” competitor DNA concentration was over-estimated, resulting in a lower competitor concentration and causing incomplete competition. However, the results of this gel shift experiment indicated that the protein of interest bound with high affinity to the NF-1 sequence. The protein of interest did not bind the sequence corresponding to the mutated oligonucleotide, indicating that the binding was specific. Table 3 gives the sequence for the consensus and mutated NF-1 oligonucleotides.

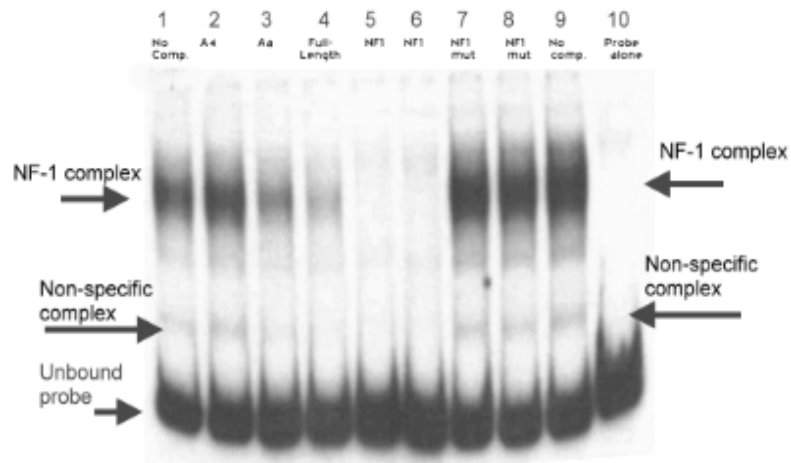


Figure 6. Competition of probe “A” with the NF-1 consensus binding sequence. This competition is lost when the binding sequence is mutated. Lanes 1 & 9= no competitor, lane 2= oligonucleotide A4 competitor (negative control), lane 3= Aa region competitor, lane 4= 150 bp “A” sequence competitor, lanes 5 & 6= NF-1 consensus competitor, lanes 7 & 8= mutated NF-1 consensus competitor.

A3 probe and NF-1 competition

In order to strengthen the argument that the transcription factor of interest was binding in the region encoded by A3 competitor, a double-stranded probe of “A3” was generated from the same oligonucleotides. When A3 was used as a competitor (Figure 7, lane 3), binding of the probe was diminished. The mutated NF-1 competitor does not compete these complexes (Figure 7, lane 5). The NF-1 consensus oligonucleotide also competed the same DNA-protein complexes when the EMSA was run with P6 and P15 cerebellar extracts (data not shown).

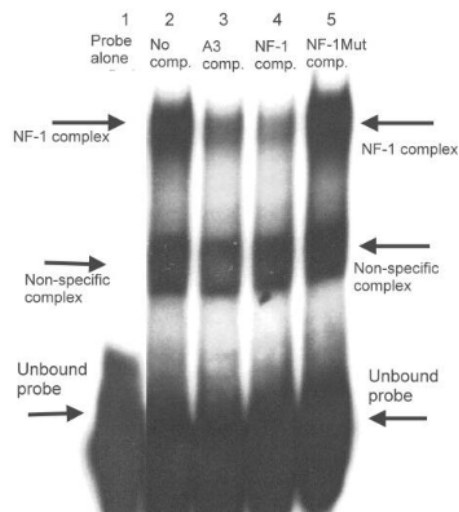


Figure 7. GCN nuclear extracts form an NF-1 complex with A3 probe that is competed by A3 oligonucleotide competitor (lane 2) and NF-1 consensus sequence (lane 3). The mutated NF-1 consensus sequence does not compete.

NF-1 Supershift

Since the results of the NF-1 competition gel shift indicated that NF-1 was indeed the binding transcription factor, a supershift experiment was carried out using two NF-1 antibodies raised against two different NF-1 isoforms from xenopus. M. Puzianowska-Kuznicka generously provided antibodies. While isoform specificity was not guaranteed, one NF-1-related antibody (α XNF-1-B1) had been previously shown to bind NF-1 proteins in a supershift, while the other (α XNF-1C) did not result in a supershift (Figure 8) (Puzianowska-Kuznicka and Shi 1996). As shown in Figure 8, α XNF-1-B1 also bound to DNA-protein complexes from probe A and GCN 6 DIV nuclear extract, resulting in the supershift seen in lane 2. By comparison α XNF-1-C1 did not supershift the complexes (lane 3). A very faint upper band discernible in lane 3 was considered artifact.

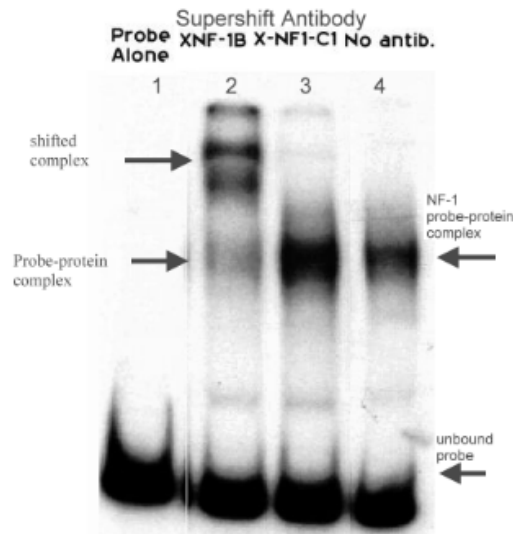


Figure 8. Supershift assay showing shift of probe A with XNF-1B antibody (lane 2). No shift was observed with XNF-1-C1 antibody (lane 3) or no antibody (lane 4).

Time Course for NF-1 complexes

The expression of the GABA_A- α 6 subunit gene promoter is not detected in GCN cultures until 6 DIV (Daniel Kilpatrick, personal communication). Since NF-1 was a likely factor involved in regulating GABA_A- α 6, a clearer understanding of the timing of NF-1 expression during development was needed. In order to determine whether NF-1 is present in the days preceding 6 DIV, a time course analysis was carried out using the full-length “A” probe and nuclear extracts from GCN 0 DIV, 2 DIV, 4 DIV and 6 DIV. The DNA-protein complexes formed in the GCN extracts from 0 to 6 DIV were essentially equivalent, indicating the presence of NF-1 in extracts from cells several days before GABA_A- α 6 expression is seen (data not shown). The commercial NF-1 consensus oligonucleotide competed the probe “A” complex at all time points, and the mutated oligonucleotide did not compete (data not shown). A mutated oligonucleotide competitor of the A3 region was also created in order to compare the effect of NF-1 competition to

that of the wild type A3 region (see Table 2 in Methods). Competition was also lost when using A3_{mut}, confirming that the A3 region contained a sequence necessary for binding (data not shown).

Figure 9 shows the GCN extracts for 0 DIV, 2 DIV, 4 DIV, and 6 DIV complexed with probe A3. As was seen with the full-length “A” probe, this gel shift experiment shows that the DNA-protein complexes seen with 6 DIV extracts are essentially the same in intensity at the earlier time points as the 6 DIV. As seen using full-length “A” probe, when the A3 probe was used both the A3 and NF-1 consensus oligonucleotide competitors also compete for binding at 0, 2, and 4 DIV, whereas the NF-1 mutant competitor does not (data not shown).

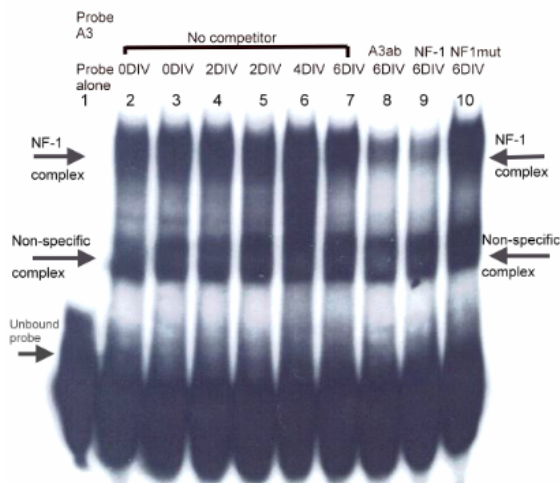


Figure 9. Time course with A3 probe and GCN nuclear extracts, and NF-1 competitors. Lanes 2 & 3= 0 DIV, lanes 4 & 5= 2 DIV, lane 6= 4 DIV, lanes 7, 8, 9, & 10= 6DIV with various competitors.

Cell Specificity of complex

NF-1 is known to regulate genes in many cell types, and therefore the next question to answer was the cell specificity of the probe-protein complexes observed. Figure 10 shows a comparison using nuclear extracts from postnatal day 6- and 15 cerebellum and postnatal day 15 cortex and probe A3. The DNA-protein complexes

observed in the lanes from day 6- and 15 cerebellar extracts appear to migrate to similar positions but are more abundant than those complexes formed with the extracts from P15 cortex, which have a lower intensity. This difference is also evident in DNA-protein complexes with probe A that are formed when using nuclear extracts from adult cerebellum and adult cortex (data not shown). The nuclear extracts were derived from whole tissue, and the possibility of complexes forming from cell types other than GCNs cannot be excluded. This experiment will eventually need to be repeated using cultured cortical neuron extracts.

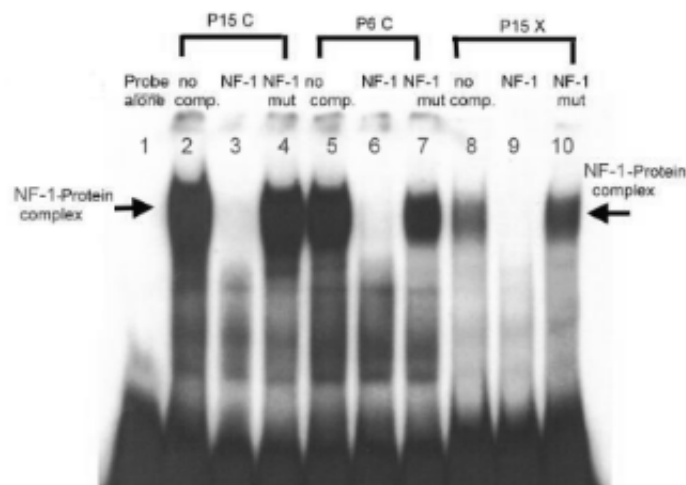


Figure 10. Probe A3 and DNA-binding protein complexes with cerebellar and cortical nuclear extracts. Lanes 2-4= post-natal day 15 cerebellum, lanes 5-7= post-natal day 6 cerebellum, lanes 8-10= post-natal day 15 cortex. Each series is shown with no competitor, and competed with NF-1 consensus and mutated NF-1 consensus sequences.

Northern Analysis

Northern analysis of GCNs was another way to examine the molecular mechanisms in the GABA_A-α6 gene activation. If NF-1 was required for GABA_A-α6 subunit gene activation, the expectation would be that RNA for NF-1 would be localized

in GCNs as well. Probes for four NF-1 isoforms were generated and a time-course analysis using RNA isolated from 0 to 6 DIV GCNs was carried out (Figure 11). As seen by ethidium bromide staining, the 6 DIV RNA extracts were partially degraded for all the blots except NF-1A. Therefore, no conclusions could be drawn from the 6 DIV in these cases. The sizes of the bands seen here were consistent with previously published findings (Chaudhry et. al, 1997). Previous NF-1A Northern blots show bands at 10 kb and 5 kb. One band corresponding to 10 kb increases between 0 and 6 DIV in GCNs (Figure 11A), indicating that NF-1A increases before GABA_A- α 6 subunit gene activation and may be a specific regulatory factor. The band corresponding to 5 kb may have been obscured by the ribosomal 28S band. An NF-1B transcript was detected at 9.7 kb in agreement with previous findings (Chaudhry et. al, 1997). NF-1B also appears to increase relative to 18S rRNA in expression in Day 2 and 4 GCNs (Figure 11B), making NF-1B a possible candidate regulatory factor of the GABA_A- α 6 subunit gene. NF-1C transcripts at 7.7 kb and 4.2 kb were previously detected in adult mouse (Chaudhry et. al, 1997), but only the 7.7 kb bands were observed here. These bands were barely visible after using twice the amount of RNA run on the other blots (10 μ g versus 5 μ g) and a 1-week exposure (Figure 11C). This suggests that NF-1C is not as abundant in neonatal mouse brain as in adult. An NF-1X transcript band at 6 kb appears to increase in expression in Day 2 and 4 GCNs (Figure 11D), suggesting that this isoform is also a possible regulatory factor of the GABA_A- α 6 subunit gene.

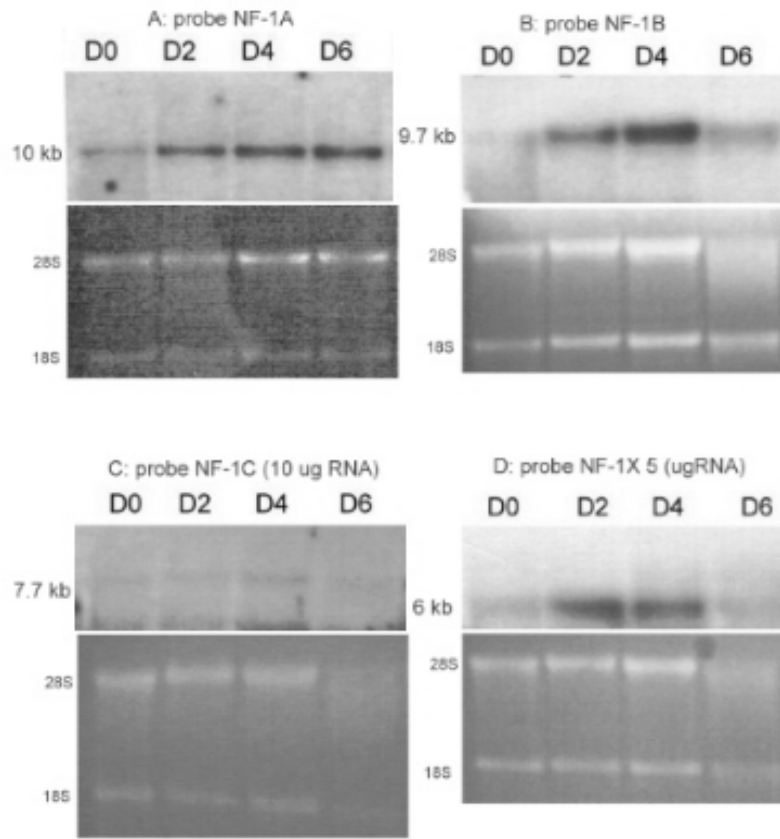


Figure 11. Northern analysis. A: shows that NF-1A increases from Day 0 to Day 6 in GCNs. B: NF-1X appears to increase in expression in Day 2 and 4 GCNs. C: Analysis of NF-1C mRNA shows very faint bands. D: NF-1B expression appears to increase strongly post Day 0, and slightly from Day 2 to Day 4.

DISCUSSION

The findings from this project implicate NF-1 as a regulatory transcription factor in the regulation of the GABA_A- α 6 subunit gene. NF-1 proteins are necessary for the proper expression of many tissue-specific and developmentally regulated genes. In addition to the four NF-1 genes (A, B, C, and X), 18 differentially spliced isoforms have been identified, although it is not known whether the isoforms are developmentally regulated or tissue specific. Little is currently known about which specific NF-1 isoforms play the most critical role in tissue-specific growth and development (Chaudhry et. al., 1997). Determining which specific NF-1 isoforms regulate the GABA_A- α 6 subunit gene may further the understanding of the GABA_A- α 6 gene promoter, as well as the mechanisms of NF-1 regulation. Cellular genes in multiple tissues have binding sites for NF-1 proteins, although few target genes for NF-1 are known in the brain (Chaudhry et. al., 1997). The genes for NF-1A, -B, -C, and -X have been previously shown to demonstrate unique expression patterns during mouse development, and their isoforms differ in their ability to activate an NF-1-dependent promoter. Because the bands observed in this project's EMSA experiments were broad, subtle changes that may exist in the complexes over the time course were not detected.

Previous transfection experiments showed that while all four murine NF-1 gene products localize to the nucleus of the infected cell, they have promoter-specific differences in their maximal activation potentials. By creating chimeric fusion constructs, previous experiments demonstrated that these differences in activation potential are regulated entirely by the NF-1 COOH-terminal regions (Chaudhry et. al, 1998). Adjacent

transcription factor binding sites in the promoter may also account for differences in activation potential. A previous study carried out with the human papilloma virus type 16 enhancer demonstrated in epithelial cells that the adjacent OCT-1 binding site was able to stabilize NF-1 binding in order enhance activation (O'Connor and Bernard, 1995). As noted before, the "A3" region of the GABA_A- α 6 promoter also contains an adjacent OCT-1 consensus site, although it is not conserved in the mouse, and has not been tested for stabilization of NF-1 activity.

Like the GABA_A receptor, the 5HT₃ receptor for serotonin belongs to the superfamily of ligand-gated ion channels. The 5HT₃ gene also has a minimal promoter that lacks a TATA-box. *In vitro* transfection of cultured cells with luciferase reporter constructs, as well as gel-shift binding assays, have shown activation of the promoter for the 5HT₃ receptor gene by binding NF-1. The results were then confirmed with supershift assays. One qualification was that the DNA-protein complex patterns observed when using extracts from cultured cells were different from those derived from primary tissue. For this thesis project, however, the results from the gel shift experiments using GCN extracts from 0 DIV cells showed similar binding patterns to those using cells at 6 DIV. NF-1 proteins alone are not likely to be the sole determinants of cell-specificity because of their enriched expression in many tissue types. However, NF-1 may combine with cofactors to regulate cell specificity by activating or silencing gene expression (Bedford et. al, 1998). One possibility is that an adjacent transcription factor, such as STAT or OCT-1 may be needed to stabilize NF-1 binding.

Northern analysis of developmental expression patterns by the Chaudhry group (1998) revealed that NF-1A is present in posterior portions of the developing brain at 9

days post-coitum (dpc). NF-1A, -B, and -X were expressed in the presumptive neocortex, ventricular zone, and the ependymal layer of the neural tube at 11.5 dpc. By 12.5- to 14.5dpc, NF-1-B and -X were highly expressed in migrating neurons of the spinal cord and cerebellum. All four NF-1 genes were expressed at birth, and in the adult cerebellar granule cells. NF-1-B expression levels were found to increase in the cortex, suggesting that it is co-expressed with NF-1-X in neurons. The preliminary results that were seen in the Northern analysis for this thesis differed somewhat from those of the Chaudhry group. In particular in 0 to 6 DIV GCNs, NF-1C RNA was only faintly detectable with a 1-week exposure to probe. The expression patterns of NF-1B and NF-1X were very similar, increasing over the 6-day time course, and agreed with the hypothesis that they are co-expressed. Because the 6 DIV RNA samples were degraded, these results were inconclusive and will need to be repeated.

The NF-1 family of transcription factors is classified as having proline-rich activation domains, and recognizing the consensus binding site, TTGGC(N₅)GCCAA. NF-1 proteins bind to DNA as both heterodimers and homodimers with the same apparent affinity. The COOH-terminal domains of the NF-1 proteins contain significant deviations, which encode transcription modulation domains (Chaudhry et. al, 1998). Variation of the COOH-terminal domains may account for the differences in activation potentials observed between alternatively spliced NF-1 proteins. Differential splicing of transcripts from each of the four NF-1 genes creates further differences among NF-1 proteins. For example, the rate of transcription may be increased by interaction of the NF-1-C isoform with the basal transcription machinery, which in turn may enhance

recruitment of the transcription apparatus components to promoters that contain NF-1 binding sites (Bedford et. al, 1998).

NF-1 proteins can also suppress nuclear oncogenes and block cell transformation (Bedford et. al, 1998). Future experiments will need to be carried out to determine the importance of NF-1 splice variants in GCNs. The results from this thesis project indicate that as many as three NF-1 gene products may be implicated in regulation of the GABA_A- α 6 gene promoter. Further time course analysis may be useful to examine whether particular NF-1 isoforms play a role in determining the cell-specificity of GCNs. Although NF-1 complexes were observed with nuclear extracts from cortex, future experiments will also need to be carried out to determine whether NF-1 mRNAs are more abundant in GCNs than in cortical cells.

One observation that was made while carrying out the EMSA experiments was that the earlier time points had a slower-moving “upper complex” that was not present with the 6 DIV extracts and is not competed by NF-1. Because NF-1 is present in the time points before GABA_A- α 6 is expressed, NF-1 is not sufficient and GABA_A- α 6 promoter requires binding of a second regulatory factor for activation to occur. One could predict that NF-1 is present and bound to the promoter, but that transcription is suppressed by another factor, perhaps the transcription factor binding this observed upper complex. Future experiments would be needed to identify the second co-regulatory factor.

SUMMARY AND CONCLUSIONS

The GABA_A- α 6 receptor subunit is a unique marker for cerebellar granule cell neurons. This thesis project utilized electrophoretic mobility shift assays, Northern analysis and transient co-transfection to look for regulatory transcription factor binding in the region proximal to the gene promoter of GABA_A- α 6. Mouse postnatal day 6 granule cell neurons were grown for 0 to 6 days *in vitro*. Granule cell neuron nuclear extracts were examined for complex formation with synthetic probes and competition assays, and RNA extracts were probed for four types of NF-1. The data identify the NF-1 transcription factor as being involved in cell-specific gene regulation.

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